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13. ABSTRACT *(Maximum 200 Words)*

Preliminary reports indicate that ultrasound contrast significantly improves the sensitivity and specificity of breast ultrasound imaging. New contrast-specific imaging modalities such as harmonic imaging (HI) may further improve the accuracy of breast ultrasound. Unfortunately, HI suffers from reduced blood-to-tissue contrast resulting from second harmonic generation and accumulation in tissue. As an alternative we propose using subharmonic imaging (SHI) by transmitting at the double the resonance frequency ($2f_o$) and receiving at the subharmonic (f_o). Because of no subharmonic generation in tissue and significant subharmonic scattering from some new contrast agents SHI has the potential to detect slow, small volume blood flow associated with tumor neovascularity, making early detection and identification of tumors very likely. Hence, the current project proposes to increase the ability of breast ultrasound to differentiate between benign and malignant lesions by combining injection of an ultrasound contrast agent with SHI.

To date, a dual-transducer pulse-echo system has been built to perform in vitro SHI measurements and initial experiments have been conducted with the contrast agents Levovist and Optison. Up to 20 dB subharmonic signal components were measured. A phantom for measuring perfusion rates is currently being designed.

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4. INTRODUCTION

The goal of any breast imaging modality is to improve the early detection of tumors and to improve the differentiation between benign and malignant lesions. While x-ray mammography is efficacious in diagnosing a high percentage of breast masses, it also produces a high rate of false positives [1]. The percentage of breast biopsies that are actually malignant vary between 10 % and 35 %. Thus, a technique that reliably differentiates between malignant and benign masses would improve the diagnosis of breast cancer and should, therefore, reduce the number of negative biopsies as well as the trauma of the patients. This proposal will attempt to establish such a technique through the novel and innovative use of subharmonic ultrasound contrast imaging.

Ultrasound imaging is currently an auxiliary modality in breast imaging. It is mainly used to differentiate between cystic and solid lesions [2]. Investigations into the possibility of breast cancer diagnosis based on Doppler ultrasound flow detection have produced mixed results, due to overlap between flow measurements in benign and malignant tumors [3-4]. One problem may be the lack of sensitivity in flow detection in small tumor vessels using ultrasound. This hypothesis is supported by reports in the pathology literature describing angiogenic vascular morphology as an independent predictor of metastatic disease [5].

Ultrasound contrast agents produce increases of 15 to 25 dB in the echo intensities of blood flow signals; especially when combined with new contrast-specific imaging modalities such as harmonic imaging [6-7]. However, harmonic imaging has been found to suffer from reduced blood-to-tissue contrast resulting from second harmonic generation and accumulation in tissue. As an alternative we propose using subharmonic imaging (SHI) by transmitting at the double the resonance frequency ($2f_o$) and receiving at the subharmonic (f_o). SHI has the potential to detect slow, small volume blood flow associated with tumor neovascularity, making early detection and identification of tumors very likely. SHI should have much better lateral resolution due to the higher transmitting frequency and should allow tumor perfusion, a measure of angiogenesis, to be estimated via time-dependent subharmonic fractional blood volume estimates. Hence, the current project proposes to increase the ability of breast ultrasound to differentiate between benign and malignant lesions by using SHI.

Quantifiable parameters of tumor angiogenesis will be estimated from the subharmonic signal intensities. A pulse-echo system will be built to perform SHI and tested in vitro as well as in vivo (in animals). The ability of SHI to depict normal vascularity as well as tumor angiogenesis will also be assessed in rabbits. Currently, the NIH and DOD have funded a study at Thomas Jefferson University into the efficacy of ultrasound contrast in the diagnosis of breast disease. We propose to expand on that project by adding SHI in the third year of this proposal. Not only is the potential of SHI in itself innovative, but because of the NIH/DOD funded study it will be possible to compare a number of new and unique approaches to breast cancer diagnosis i.e., SHI, 2D power Doppler with and without contrast as well as harmonic imaging directly to x-ray mammography. Furthermore, this project is extremely cost-effective because the existing grants coves a majority of the personnel costs as well as all major equipment purchases. The amalgamation of the NIH/DOD project with the current proposal also allows for basic research

into the correlation between SHI flow signals and pathologically detected lesion vascularity. This will enable a deeper understanding of the relationship between tumor neovascularity and ultrasound flow measurements

Consequently, this project proposes the development of a novel contrast specific imaging mode called SHI and the derivation of quantitative tumor angiogenesis estimates from SHI data. The fundamental hypothesis is that the neovasculature of malignant lesions can be visualized and quantified with SHI, thus, improving the diagnosis of breast cancer.

5. BODY

The central hypothesis of this project is that the differentiation between benign and malignant breast lesions can be improved by detection and estimation of tumor neovascularity using contrast enhanced SHI. To investigate this hypothesis SHI will be investigated in vitro and then in vivo in rabbits with VX-2 tumors. Finally, approximately 50 women with breast lesions will be recruited in year three and imaged using contrast enhanced SHI. The specific tasks of the project (as presented in the original Statement of Work) can be found in Appendix I.

First an outline of the methods applied will be given followed by a presentation of the results to date. Finally, the conclusions and future directions of the research will be discussed.

5.1 Methods

In Vitro experiments

A pulse-echo system was built to perform SHI and measure the FBV as a function of time (Fig. 1). The setup consists of a pair of confocally positioned broadband focused transducers (diameter: 2.54 cm). Transmit/receive transducer pairs of 3.5/3.5 MHz or 5.0/2.25 MHz were employed. This substantially improved the spatial resolution of the system, because scattered signals only come from the microbubbles in the small confocal region of the two transducers (1-4 mm^3 for 2 MHz transmission). This flow system was set up to test SHI as well as to determine the optimal acoustic conditions for SHI. Insonation frequencies of 4.0 and 4.4 MHz were used (since this is the frequency range used in the ultrasound scanner employed for our preliminary SHI work [8]). The input voltage was varied from 0.025 to 10 Vp-p with CW and PW insonation. The latter was studied with PRF's from 1 to 80 kHz and pulse lengths from 16 to 40 cycles. The received echo was averaged on a LeCroy Oscilloscope (Chestnut Ridge, NY) for 100 times and then read on to a PC. Analysis was done on the PC using Matlab (The Mathworks Inc, Nantick, MA). The sampling frequency was 20 MHz.

In the original proposal efforts within the first year were also aimed towards a tubular phantom to simulate capillary flow and the flow velocity were to be controlled within the capillary flow velocity range (0.1-10 mm/s) using a precision flow pump capable of generating constant and pulsatile flow profiles. Measured perfusion rates were to be compared to the given volume flow rate. This phantom is currently being designed (appropriate tubing with an inner diameter of 230 μm has been identified) and perfusion experiments will commence shortly under task 1c.

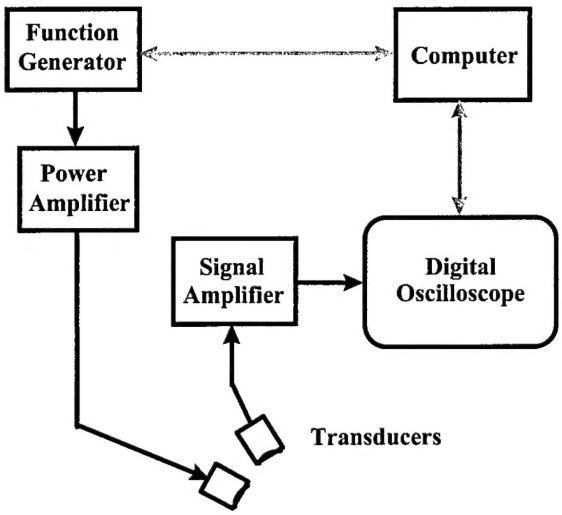

Figure 1. *The dual-transducer pulse-echo system built to perform SHI. A function generator will produce a sequence of transmit pulses which will first be amplified and then supplied to a single-element broadband focused transducer. Another broadband focused transducer (confocally positioned to the first transducer) will sense signals scattered from the contrast bubbles. The received signals, after being amplified, will be digitized using a digital oscilloscope. The digitized signals are further processed to obtain the subharmonic amplitude (with a bandpass filter) using LabView® (National Instruments, Austin, TX).*

5.2 Results and Discussion

An important part of this project requires the hiring of a student. However, no one suitable could initially be found due to the start date of this project (most students are committed by mid-summer). Hence, it was not until January 2001, that Govind Bhagavatheeshwaran was employed as the student on this grant. This research project will now form the main part of his Master of Science degree (to be obtained from Drexel University with P.M. Shankar and F. Forsberg as his supervisors). Nonetheless, the project was delayed by approximately six months relative to the original plans.

A dual-transducer pulse-echo system was built (as outlineed in Figure 1) to perform SHI. Initial experiments were conducted with Levovist (Schering AG, Berlin, Germany). This agent was chosen merely due to availability and to provide the student with a contrast agent on which to gain experience. An example of subharmonic generation from Levovist obtained with CW excitation is given in Figure 2. Notice, that no subharmonic components were detectable with transmit amplitudes less than 4 Vp-p and that the maximum SHI amplitude measured was 20 dB (for voltages > 8 Vp-p).

Once the first version of software for SHI measurements had been constructed (as part of task 1a) subsequent experiments were conducted with the contrast agent Optison (Mallinckrodt, St Louis, MO), since it has already been selected for the human studies and is know to have good

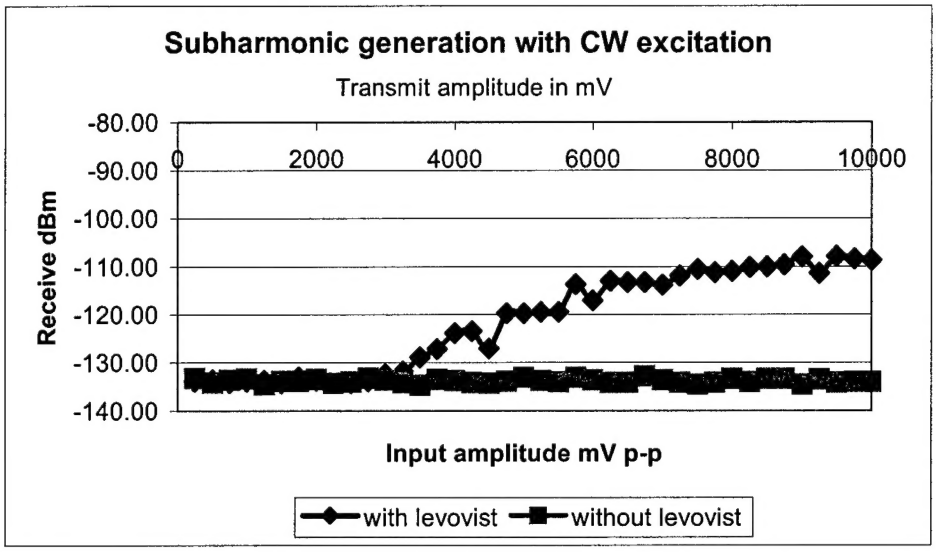

Figure 2. *Subharmonic signal components obtained from Levovist with 4.4 MHz CW excitation.*

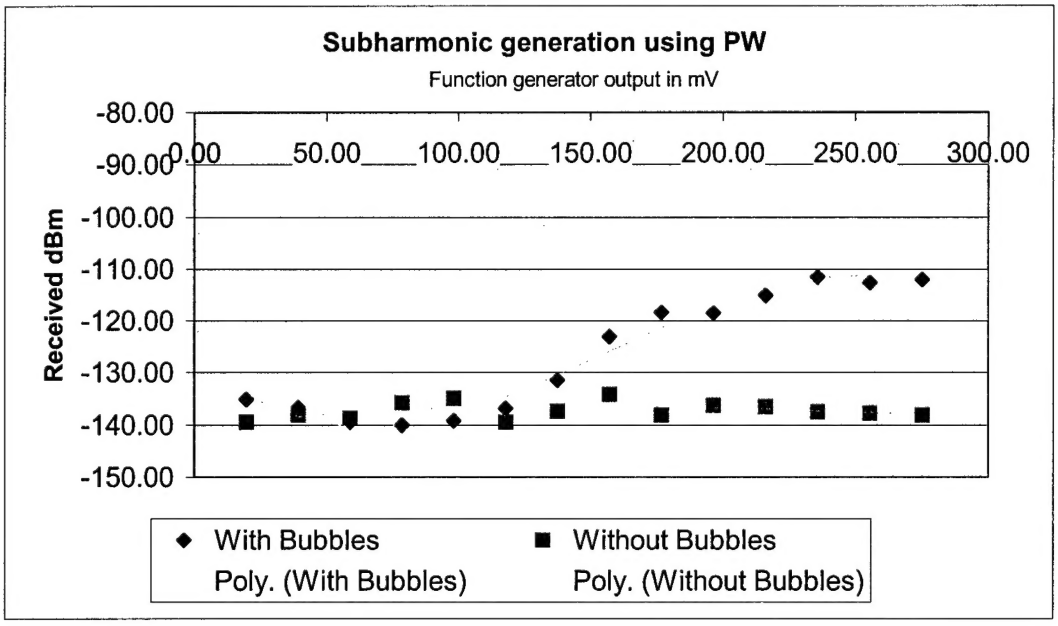

Figure 3. *Measurement of subharmonic signals from Optison using 4 MHz PW excitation at a 1 kHz PRF.*

subharmonic properties [8-9]. Optison is approved for use in echocardiography by the U.S. Food and Drug administration (for improved endocardial border delineation). It consists of a suspension of perfluoropropane-filled albumin microspheres with a concentration of 6.3×10^8 bubbles/ml and the bubbles have mean diameters in the range of 3 to 5 μm. An example of the

subharmonic signal components measured with Optison is presented in Figure 3 along with a least-squares polynominal fit to the data. Up to 20 dB of subharmonic signals were measured similarly to Figure 2, but these results were obtained with a PRF of 1 kHz in PW mode which makes direct comparisons difficult. However, the results are similar to previous work by our group [9]. Optimal acoustical imaging parameters for SHI with Optison were explored within the parameter space described in Section 5.1. fulfilling task 1b.

6. KEY RESEARCH ACCOMPLISHMENTS

- A dual-transducer pulse-echo system was built to perform SHI.
- Initial experiments were conducted with Levovist and Optison.
- Optimal acoustical imaging parameters for SHI were explored.

7. REPORTABLE OUTCOMES

Govind Bhagavatheeshwaran has been employed on this project and is working towards his Master of Science degree in Biomedical Engineering (to be obtained from Drexel University) with P.M. Shankar and F. Forsberg (the PI) as his supervisors.

8. CONCLUSIONS

A dual-transducer pulse-echo system was built to perform in vitro SHI measurements and initial experiments were conducted with the contrast agents Levovist and Optison. Up to 20 dB subharmonic signal components were measured. A phantom for measuring perfusion rates is currently being designed.

In summary, task 1b has been completed while tasks 1a and 1c are ongoing, but due to the delay in hiring a student the project is approximately 6 months behind the original schedule.

9. REFERENCES

1. Feig SA: Breast masses: Mammographic and sonographic evaluation. *Radiologic Clin North Am* 30:67-92, 1992.
2. Jackson VP: The Role of US in Breast Imaging. *Radiology*, 177:305-311, 1990.
3. Bohm-Velez M, Mendelson EB: Computed tomography, duplex Doppler ultrasound and magnetic resonance imaging in evaluating the breast. *Semin Ultrasound CT MR*, 10:171-176, 1989
4. Adler DD, Carson PL,. Rubin JM, Quinn-Reid D: Doppler ultrasound color flow imaging in the study of breast cancer: preliminary findings. *Ultrasound Med. Biol.*, 16: 553-559, 1990.
5. Weidner N, Folkman J, Pozza F, et al. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast cancer. *J Natl. Cancer Inst.,* 84: 1875-1887, 1992.
6. Forsberg F, Merton DA, Liu JB, Needleman L, Goldberg BB: Clinical applications of ultrasound contrast agents. *Ultrasonics*, 36: 695 - 701, 1998.
7. Goldberg BB, Raichlen JS, Forsberg F. *Ultrasound Contrast Agents: Basic Principles and Clinical Applications* (2nd Ed). Martin Dunitz Ltd., England, 2001.
8. Shi WT, Forsberg F, Hall AL, Chiao RY, Liu JB, Miller S, Thomenius KE, Wheatley MA, Goldberg BB: Subharmonic imaging with contrast agents: initial results. *Ultrasonic Imaging*, 21: 79 - 94, 1999.
9. Forsberg F, Shi WT, Hall A, Thomenius KE, Goldberg BB: Comparison of nonlinear US imaging modes using contrast agents. *Radiology* 209(P):190 (Abstr.), 1998.

Appendix I

The Statement of Work from the original proposal:

Objectives 1 - 2
Task 1: Software development and *in vitro* experiments (months 1 - 24)

a. Develop software for SHI and for FBV estimates to be produced from SHI data (months 1 - 24).
b. Design and implement pulse echo SHI setup (months 1 - 6).
c. Perform *in vitro* flow phantom experiments comparing SHI and FBV estimates to absolute perfusion and flow rates (months 6 - 12).

Objectives 2 - 3
Task 2: Animal experiments and data collection (months 13 - 24)

a. Perform *in vivo* experiments in 12 normal rabbits comparing FBV estimates to absolute flow rates and perfusion obtained with colored microspheres (months 13 - 20).
b. Perform *in vivo* experiments in 6 rabbits with renal VX-2 tumors implanted comparing FBV estimates to absolute tumor perfusion obtained with colored microspheres (months 20 - 24).
c. Evaluate the performance of SHI in the detection of rabbit VX-2 tumors compared to conventional ultrasound imaging, with and without contrast administration, as well as to harmonic imaging (months 13 - 24).

Objectives 4 - 5
Task 3: Human data collection and analysis (months 25 - 36)

a. Recruit 50 - 75 patients, which is about two-thirds of the anticipated number of patients being enrolled in the existing NIH/DOD supported contrast study (months 25 - 36).
b. Perform SHI contrast studies as part of the already funded NIH/DOD project. This involves an extra injection of contrast (within the permitted total dose) and will add no more than 20 minutes to the total duration of the contrast study (months 25 - 36).
c. Research coordinator to collect clinical information, pathology results, etc. (months 25 - 36).
d. Incorporate SHI findings into the existing database developed for the NIH/DOD supported study (months 25 - 36).
e. Perform ROC analysis in collaboration with the statistician (months 30 - 36).
f. Perform remaining statistical analysis in collaboration with the statistician (months 30 - 36).